Flavin Thermodynamics Explain the Oxygen Insensitivity of Enteric Nitroreductases†

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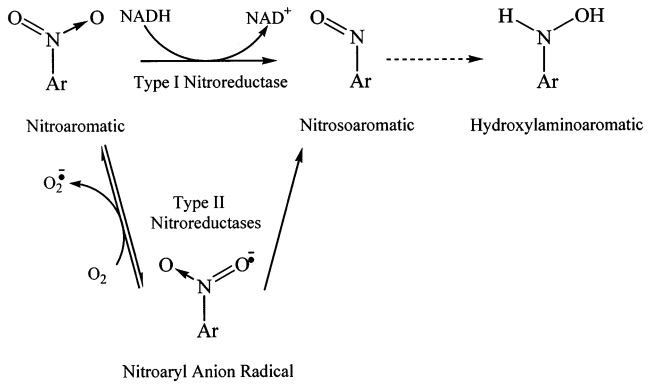
ABSTRACT: Bacterial nitroreductases are NAD(P)H-dependent flavoenzymes which catalyze the oxygen-insensitive reduction of nitroaromatics, quinones, and riboflavin derivatives. Despite their broad substrate specificity, their reactivity is very specific for two-electron, not one-electron, chemistry. We now describe the thermodynamic properties of the flavin mononucleotide cofactor of *Enterobacter cloacae* nitroreductase (NR), determined under a variety of solution conditions. The two-electron redox midpoint potential of NR is −190 mV at pH 7.0, and both the pH dependence of the midpoint potential and the optical spectrum of the reduced enzyme indicate that the transition is from neutral oxidized flavin to anionic flavin hydroquinone. The one-electron-reduced semiquinone states of both the free enzyme and an NR−substrate analogue complex are strongly suppressed based on optical spectroscopy and electron paramagnetic resonance measurements. This can explain the oxygen insensitivity of NR and its homologues, as it makes the execution of one-electron chemistry thermodynamically unfavorable. Therefore, we have established a chemical basis for the recent finding that a nitroreductase is a member of the *soxRS* oxidative defense regulon in *Escherichia coli* [Liochev, S. I., Hausladen, A., Fridovich, I. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96* (7), 3537−3539]. We also report binding affinities for the FMN cofactor in all three oxidation states either determined fluorometrically or calculated using thermodynamic cycles. Thus, we provide a detailed picture of the thermodynamics underlying the unusual activity of NR.

Nitroreduction is the initial step in the catabolism of a variety of structurally diverse nitroaromatic compounds (*3*). The enzymes that catalyze this chemistry are termed nitroreductases and have been grouped into two categories (*4*): oxygen-insensitive or type I nitroreductases catalyze the pyridine nucleotide-dependent reduction of nitroaromatics to either a hydroxylamino- or aminoaromatic endproduct by two-electron steps, while oxygen-sensitive or type II nitroreductases catalyze the reduction of nitroaromatics by one-electron steps utilizing a variety of electron donors. The oxygen sensitivity of the latter nitroreductases derives from the ready reoxidation of the nitro anion radical product by oxygen, forming a superoxide radical and regenerating the original nitroaromatic compound. This "futile cycle" can cause oxidative stress by producing large amounts of superoxide (see Scheme 1.) Enzymes with oxygen-sensitive nitroreductase activity include aldehyde oxidase (*5*), cytochrome *c* reductase (*6*), cytochrome P-450 reductase (*7*), glutathione reductase (*8*), succinate dehydrogenase (*9*), thioredoxin reductase (*10*), and xanthine oxidase (*11*), while enzymes with oxygen-insensitive nitroreductase activity include DT diaphorase (*12*), xanthine dehydrogenase (*13*), and the classical nitroreductase enzymes from enteric bacteria (*10*, *14*). The classical nitroreductase from *Escherichia coli* has recently been shown to be induced during oxidative stress conditions, as a member of the *soxRS* regulon (*15*, *16*), an indication that this enzyme protects against oxidative stress rather than generating it. Despite the large body of literature on both oxygen-sensitive and oxygen-insensitive nitroreduc-

Scheme 1: Summary of Reductive Metabolism of Nitroaromatics Catalyzed by Nitroreductases



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tases, the physiochemical basis for the difference between the two types of activity has not been established.

Enterobacter cloacae nitroreductase (NR)[1] is a classical nitroreductase which catalyzes the NAD(P)H-dependent reduction of nitroaromatics, riboflavin derivatives, and quinone compounds using a tightly-bound flavin mononucleotide (FMN) cofactor (*17*−*20*). It was originally characterized by Bryant et al. (*14*) and cloned from an *Enterobacter* strain selected from a weapons dump for its ability to degrade nitroaromatics. It is now known to be a member of a larger family of proteins found in bacteria and archea (*21*). Bryant et al. have shown NR to be an oxygen-insensitive nitroreductase, and the NADH oxidase activity of NR has been determined to be very low, having a k_{cat} of 0.11 s^{-1} in the presence of saturating NADH and with the product of oxygen reduction being hydrogen peroxide, not superoxide (*17*, *19*). NR reduces nitrobenzene to a hydroxylaminoaryl endproduct (*19*), and homologues of NR have been implicated in drug resistance in *Helicobacter pylori* and several *Clostridium* species (*22*, *23*) as well as in herbicide resistance in the cyanobacterium *Synechocystis* sp. PCC 6803 (*24*). The *E. coli* homologue of NR is being developed for use in gene therapies as a suicide gene in combination with the nitroaromatic prodrug CB 1954 (*25*−*28*), and nitroreductases are possible catalytic agents for nitroaromatic bioremediation (*3*). In light of the interesting and practical roles mediated by these enzymes, it is important to obtain a full understanding of their structure and mechanism so that they can be manipulated and/or inhibited.

Our laboratories have been engaged in the biochemical and biophysical elucidation of the mechanism and properties of NR (*17*, *19*, *29*−*31*). We have previously described the kinetic mechanism and specificity of the enzyme, as well as the X-ray crystal structures of two oxidized enzyme−inhibitor complexes and the fully reduced enzyme. We now describe the thermodynamics of the bound FMN cofactor of NR, which to yield NR, which place fundamental limits on this enzyme's catalytic action. Thus, NR's reduction potentials provide an explanation for its identity as an oxygen-insensitive nitroreductase and, as discussed below, may offer a general basis for the oxygen insensitivity of all enteric nitroreductases.

MATERIALS AND METHODS

Chemicals. Phenosafranin (PS, C.I. #50200), purchased from Aldrich, was purified chromatographically using silica gel (Sigma) with 4:1 benzene:methanol as the eluant. PS concentrations were determined spectrophotometrically (ϵ_{520} = 42.3 mM^{-1}) (*32*). FMN was purified by the HPLC method of Light et al. (*33*), lyophilized, and stored at −20 °C in the dark until use. Piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) was from Research Organics. Deazaflavin 3-sulfonate (D3S) was the kind gift of the late Dr. Vincent Massey of the Department of Biological Chemistry, University of Michigan. Oligonucleotides were purchased from Integrated DNA technologies (Corralville, IA).

Enzymes. NR was prepared and assayed as described (*29*). The preparations used had a specific activity of 582 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. NR concentrations were determined spectrophotometrically using ϵ_{280} = 66.5 mM^{-1} (*29*). A gene encoding the P2A mutant of *Desulfovibrio vulgaris* flavodoxin (FD) was designed using the program Amplify (*34*). The gene was constructed using assembly PCR (*35*) and ligated into the overexpression vector pET 24d(+) (Novagen, Inc.) to yield the plasmid pRKFD. The plasmid was transformed into DH5α cells and sequenced in the region of interest to verify the presence of the intact FD gene. For expression, the plasmid was transformed into BL21(DE3) cells, grown in TPP medium (*36*) at 37 °C to an OD_{600} of 1.0, and induced with IPTG at a final concentration of 1 mM for 2 h before harvesting. Expressed FD was purified by the method of Krey et al. (*37*), resulting in a final yield (calculated spectrophotometrically using ϵ_{457} = 10.7 mM^{-1}) of 16 mg of purified FD per liter of TPP media.

Anaerobic Titrations. All titrations were performed in the anaerobic apparatus described previously (*29*, *38*, *39*). Solutions were made anaerobic by repeated cycles of evacuation and flushing with argon.

Stoichiometric titration solutions consisted of 90−95 μM NR in a 5 mL volume of 100 mM PIPES, 50 mM KCl, 0.02% NaN_3, pH 7.0, either with or without 1 μM methyl viologen (MV). Titrants consisted of either dithionite (DT) in 100 mM KOH, calibrated against oxidized FMN (FMN_{ox}) or NAD^+, NADH, or thio-NADH in 100 mM PIPES, 50 mM KCl, 0.02% NaN_3, pH 7.0, calibrated spectrophotometrically (FMN_{ox} λ_{max} = 445, ϵ_{445} = 12 600 M^{-1} cm^{-1}, NADH λ_{max} = 340, ϵ_{340} = 6220 M^{-1} cm^{-1}, thio-NADH λ_{max} = 395, ϵ_{395} = 11 300 M^{-1} cm^{-1}). Titrant solutions were made anaerobic in sealed serum stopper vials and stored on ice for the duration of each experiment (less than 16 h). Small amounts of cold titrant (<20 μL) were injected through one port of the apparatus, and the approach of the reaction to equilibrium was monitored optically at 454 nm. Visible region absorbance spectra were collected for each titration point after the solution had reached equilibrium.

A control experiment was performed to assess the possibility of a covalent interaction between the DT oxidation product, sodium sulfite, and oxidized NR (*40*). No spectral perturbation could be detected after incubation of NR with 1 mM sodium sulfite at 25 °C for 1 h, indicating that NR does not form the flavin−sulfite adducts typical of some flavoproteins and therefore does not interact with DT breakdown products (*41*).

Photoreduction experiment solutions consisted of 60−65 μM NR in a 5 mL volume of 2.4 μM D3S, 10 mM EDTA, 100 mM PIPES, 50 mM KCl, 0.02% NaN_3, pH 7.0, either with or without 1 μM MV (*42*). Photoreduction was achieved by irradiating the solution with a Kodak model 4600 carousel projector (Kodak Inc., Rochester, NY) for the indicated time periods. After each irradiation time point, visible absorbance spectra were collected immediately, and photoreduction was resumed.

Potentiometric titration solutions consisted of 85−90 μM NR in a 5 mL volume of 7 μM PS, 1 μM MV, 100 mM

[1] Abbreviations used: BA, benzoic acid; D3S, deazaflavin 3-sulphonate; DT, dithionite; E_1, one-electron reduction potential for the oxidized flavin−flavin semiquinone redox pair; E_2, one-electron reduction potential for the flavin semiquinone−flavin hydroquinone redox pair; E_m, two-electron reduction potential; HAB, hydroxylaminobenzene; NB, nitrobenzene; NHE, normal hydrogen electrode; NR, *Enterobacter cloacae* nitroreductase; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; PS, phenosafranin; thio-NADH, thionicotinamide adenine dinucleotide, reduced form.

PIPES, 50 mM KCl, 0.02% NaN_3, adjusted to the specified pH. When indicated, the specified concentration of benzoic acid was added as a concentrated stock solution in the same buffer. An MI-800-410 combination Pt and $Ag/AgCl_2$ microelectrode (Microelectrodes Inc., Bedford, NH) was used to continuously monitor the solution reduction potential. PS and MV served only as redox buffers and to mediate electron transfer between NR and the electrode, not as redox indicators. Small volumes (<20 μL) of DT solution, prepared and maintained as described above, were injected, and the approach of the solution to redox equilibrium was monitored potentiometrically and spectrophotometrically. Visible region absorbance spectra were collected for each titration point, after the solution had reached equilibrium, and the fractional concentration of oxidized PS was determined spectrophotometrically at 520 nm. This value was used to calculate and subtract the contribution of oxidized PS to the solution absorbance at 454 nm ($\epsilon_{454} = 10.1\ mM^{-1}\ cm^{-1}$), with the remainder being due to oxidized NR. The absorbance data were plotted as a function of the reduction potential at equilibrium. The data were fit with the Nernst equation with allowance for 2 electrons per redox event:

$$E = E^\circ + 29.5\ mV \log\left(\frac{Ox}{Ox_{max} - Ox}\right) \qquad (1)$$

where E is the ambient potential in millivolts measured vs the normal hydrogen electrode (NHE), Ox is the absorbance of oxidized NR or PS, Ox_{max} is the maximal change in absorbance, and E° is the fitted reduction midpoint potential. After reduction was complete, the anaerobic apparatus was opened briefly to the atmosphere and allowed to re-equilibrate to test the titration for reversibility. For each set of solution conditions, a control titration of PS in the absence of NR was performed to test for interactions between the enzyme and redox dye (*43*). The upper limit of the experimental error in these determinations is ±5 mV, based on experimental reproducibility and the standard error of the fit.

Electron Paramagnetic Resonance (*EPR*). EPR spectroscopy was performed using a Bruker model ESP300E spectrometer with an Oxford ESR model 900 continuous-flow cryostat. Frequency was measured by a Hewlett-Packard model 5350B frequency counter. Each spectrum was the sum of eight 168-s scans collected at 20 K, 201 μW, and 9.4285930 kHz with 5.069 G modulation amplitude at 100 GHz. All protein solutions were in 50 mM KH_2PO_4, 50 mM KCl, 0.02% NaN_3, pH 7.0. FD samples consisted of 10−30 μM FD and included 2 μM anthraquinone 2,6-disulfonate ($E_{m,7} = -184$ mV), and 1 μM MV ($E_{m,7} = -430$ mV) as mediators. The NR sample contained 341 μM and the mediators PS ($E_{m,7} = -245$ mV) and MV at 1 μM each. All samples were poised at their E_m value (−190 mV vs NHE for NR, −291 mV for FD) using DT, transferred to a Suprasil quartz EPR tube by cannula under argon, and flash frozen. Samples were stored briefly in liquid nitrogen until the EPR spectra were recorded. Control spectra of identical solutions poised at the same potentials in the absence of the proteins were taken to evaluate the possibility of mediator interference.

Preparation of ApoNR. ApoNR was generated by a modification of the method of Van Berkel et al (*44*). Briefly, holoNR (28.3 mg) in 1.7 M ammonium sulfate, 1 M KBr, 50 mM KH_2PO_4, 0.02% NaN_3, pH 7.0, was applied to a 2.5- × 5-cm column of Phenyl Sepharose (Sigma) at room temperature. FMN_{ox} was removed by elution with the same buffer, adjusted to pH 5.0. ApoNR was then eluted using 50 mM phosphate buffer, pH 7.0, and immediately cooled to 4 °C. Yields were typically above 50%. Reconstitution experiments were performed by incubating a small volume (less than 200 μL) of approximately 30 μM apoNR with an equal volume of 10 mM FMN_{ox} in 50 mM phosphate buffer, pH 7.0, at 4 °C for 30 min and then assaying the resultant solution for activity as described previously (*29*). ApoNR absorption coefficients were determined using the guanidine·HCl denaturation method of Edelhoch (*45*, *46*). Activity measurements on FMN-reconstituted apoNR give the same specific activities, within error, as the starting material. Thus, the protein recovered does not appear to have been damaged in any way.

FMN Binding Studies. NR's affinity for one FMN per monomer was measured fluorometrically. Fluorescence-monitored binding measurements were carried out at 25 °C, utilizing an SLM 8000 spectrofluorometer (SLM-Aminco, Rochester, NY) equipped with thermospacers and a circulating water bath. Excitation was achieved with a xenon arc lamp through a double monochromator set at 454 nm and a vertical polarizer. Emission was observed through a monochromator and a polarizer set at 54.7° to the vertical (the magic angle). Both monochromators had a resolution of 4 nm full width at half-maximum. Small aliquots (<10 μL) of cold apoNR were titrated into 2.5 mL solutions containing various concentrations of FMN_{ox} in 100 mM PIPES, 50 mM KCl, 0.02% NaN_3, pH 7.0. Each solution was allowed to stir for at least 5 min after each addition before data collection was initiated. The fluorescence quenching data were fit using the program SYSTAT (SPSS, Inc.) with eq 2, which accounts for tight-binding effects (*43*):

$$\Delta F = \left[\frac{K_d + E_T + [FMN]_T - \sqrt{(K_d + E_T + [FMN]_T)^2 - 4E_T[FMN]_T}}{2E_T}\right] \qquad (2)$$

where ΔF is the change in fluorescence emission at 520 nm, ΔF_T is the change in fluorescence at this wavelength at saturating ligand concentrations, K_d is the dissociation constant obtained for the apoNR−FMN_{ox} complex, $[FMN]]_T$ is the total FMN_{ox} concentration, and E_T is the total concentration of NR.

Analytical Ultracentrifugation. To measure NR's dimerization affinity as a function of FMN availablility, equilibrium ultracentrifugation experiments were carried out at 20 °C in a Beckman Optima XL-I analytical ultracentrifuge. Six-channel, 1.2-cm charcoal-filled Epon centerpieces were used throughout. Samples of apoNR and holoNR were dialyzed in 100 mM PIPES buffer, 50 mM KCl, 0.02% NaN_3, pH 7.0. Loading concentrations were 30, 10, and 3.0 μM, and equilibrium protein concentration distributions were measured utilizing the protein absorbance at 280 nm. All centrifuge runs were carried out initially at 20 krpm for at least 24 h, after which data were acquired at 3-h intervals until no detectable changes in concentration distribution were

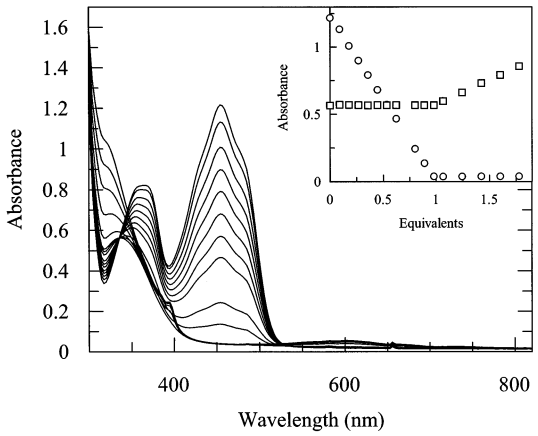

FIGURE 1: Anaerobic dithionite titration of NR. A 5 mL solution containing 112 μM NR in 100 mM PIPES buffer, 50 mM KCl, 5 μM methyl viologen, 0.02% NaN_3, pH 7.0, at 25 °C was titrated with small volumes (<20 μL) of cold DT in 100 mM KOH which had been precalibrated vs FMN. The small absorbance peaks at 390 and 600 nm are reduced MV which did not appear until complete NR reduction had occurred. (Inset) Absorbance at 336 nm, the isosbestic point (□), and 454 nm, the λ_{max} of oxidized NR (○) plotted vs equivalents of dithionite. The invariant absorbance at 336 nm and the linear absorbance decrease at 454 nm during the titration indicate that only two chemical species are present during the course of the titration, oxidized and two-electron-reduced NR.

observed. Subsequently, after equilibrium had been established, data were collected at 24 and 34 krpm.

Centrifuge data were fit globally using the program NONLIN (*47*) with either a nonassociating (one molecular species) or an associating model according to eqs 3 and 4, respectively (*48*):

$$A_r = A_0\, e^{H\bar{M}_w(X^2 - X_0^2)} + E \quad (3)$$

$$A_r = A_0\, e^{HM_1(X^2 - X_0^2)} + (A_0)^n K_D^{-1}\, e^{HM_1 n(X^2 - X_0^2)} + E \quad (4)$$

$$H = \frac{(1 - \bar{v}\rho)\omega^2}{2RT} \quad (5)$$

where A_r and A_0 are the absorbances at radii X and X_0, respectively, M_w is the weight-average molecular weight, M_1 is the monomeric molecular weight, n is the stoichiometry, K_D is the dissociation constant, E is the baseline offset, and where υ is the partial specific volume of the protein, calculated from the sequence to be 0.7323 mL/g, ρ is the measured specific gravity of the buffer (1.0165 g/mL at 20 °C), and ω is the angular velocity of the rotor.

RESULTS

Stoichiometric Titrations of NR. Since NR reduces nitrobenzene to hydroxylaminobenzene without detectable production of free nitrosobenzene (*19*), it is important to evaluate the possibility of redox-active groups in NR in addition to the FMN. Figure 1 depicts the anaerobic reduction of NR with DT at pH 7.0, 25 °C. One two-electron equivalent of either DT or NADH completely reduces the active-site cofactor of NR. No further reducing equivalents are absorbed by other components of the enzyme, as evidenced by increasing absorbance due to DT at 336 nm (the isosbestic point for NR reduction) following the addition of reducing equivalents sufficient to complete FMN reduction. Thus, there is no evidence for a cysteine sulfenic acid or other redox active group in NR. The four-electron reduction of nitrobenzene therefore must occur in two discrete two-electron steps (i.e., two full catalytic cycles), or a second molecule of NADH in addition to the one which reduces the FMN must participate directly in the reaction.

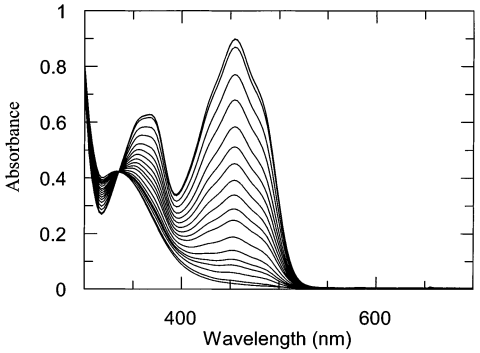

FIGURE 2: Anaerobic photoreduction of NR. A 5 mL solution containing 78.5 μM NR in 100 mM PIPES buffer, 50 mM KCl, 2.4 μM D3S, 10 mM EDTA, 0.02% NaN_3, pH 7.0, at 25 °C was irradiated with a Kodak model 4600 carousel projector for various periods of time. The isosbestic point at 336 nm again indicates that only two chemical species are present during the course of the titration, oxidized and two-electron-reduced NR.

Under the above conditions, NADH reacts with NR faster than the mixing time of the experiment (approximately 15 s) either in the presence or in the absence of 1 μM MV. DT also reacts with NR faster than the mixing time of the experiment in the presence of MV, but in the absence of MV the reaction takes several minutes to complete. Thio-NADH, an NADH analogue with a midoint potential 40 mV more positive than that of NADH, and which has been shown previously not to be a substrate for NR (*19*), reacts with the enzyme very slowly, exhibiting a second-order rate constant for NR reduction of 3.46 $mM^{-1}\ min^{-1}$ in the absence of MV (data not shown). Thus, the failure of thio-NADH as a substrate is confirmed to be kinetic as opposed to thermodynamic, and the amide functionality of NADH appears to enhance substrate binding and/or subsequent electron transfer to the bound FMN cofactor, as proposed earlier (*19*).

The presence of an isosbestic point at 336 nm and the linear decrease in absorbance at 454 nm during the DT titration of NR, even in the presence of the one-electron mediator MV (see inset, Figure 1), indicates that only two chemical species are present during the course of the titration. No formation of one-electron-reduced flavin semiquinone can be detected, indicating that this oxidation state is thermodynamically unstable under these conditions. Similar results were obtained when this experiment was repeated without MV and during the course of reductive NADH titrations either with or without MV.

Photoreduction. In the absence of mediators, the obligate one-electron nature of photoreduction by D3S can sometimes kinetically drive the flavin semiquinone oxidation state to a higher population than that allowed under equilibrium conditions (*42*). The time course of a D3S-catalyzed anaerobic photoreduction of NR in the absence of MV is depicted in Figure 2. Once again, the presence of an isosbestic point (at 336 nm) signifies the absence of any measurable flavin semiquinone formation during the course of this experiment.

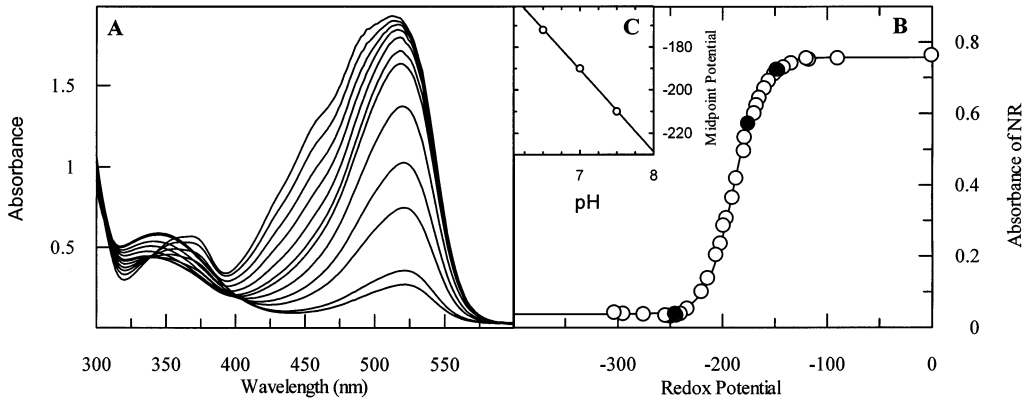

FIGURE 3: Potentiometric titration of NR. A 5 mL solution containing 68.9 μM NR and 7 μM PS in 100 mM PIPES buffer, 50 mM KCl, 5 μM MV, 0.02% NaN_3, pH 7.0, at 25 °C was titrated with small volumes (<10 μL) of cold DT in 100 mM KOH while the solution redox potential was monitored with a combination Pt and $Ag/AgCl_2$ microelectrode. (A) Visible region spectra of NR/PS mixture upon equilibration after each injection of DT. Some spectra were omitted for clarity. (B) Absorbance of NR at 454 nm plotted vs reduction potential. At each point, oxidized PS concentrations were calculated using the A_{520}, and the PS contribution to the A_{454} was subtracted. ○, Points during reductive titration; ●, return points collected as in Materials and Methods. The line is a plot of eq 4 using an E_m of −190 mV vs NHE. (C) pH dependence of E_m.

Table 1: Redox Potential Data of Nitroreductase in 100 mM PIPES buffer, 50 mM KCl, 1 μM Methyl Viologen, 0.02% NaN_3, 25 °C

		E_m (mV)[a]		
pH	[benzoate] (mM)	phenosafranin alone	phenosafranin with enzyme	enzyme
7.0	0	−245	−247	−190
6.5	0	−230	−229	−172
7.5	0	−265	−266	−210
7.0	0.5	−250	−248	−216
7.0	25	−251	−250	−243
7.0	50	−251	−251	−248

[a] All redox potentials are vs NFE. Phenosafranin titrations in the absence of NR were performed to rule out any binding of PS to NR.

Identical results were obtained with 1 μM MV present. Thus, despite a variety of attempts, we are unable to generate optically detectable flavin semiquinone under either equilibrium (DT and NADH titrations with MV) or kinetic trapping conditions (photoreduction without MV), despite the high extinction coefficient expected for a flavin semiquinone (*40*).

Reduction Midpoint Potential Titrations. The two-electron reduction midpoint potential, or E_m, of NR in pH 7.0 PIPES buffer is −190 mV vs NHE (see Figure 3). Control experiments using a variety of redox mediators demonstrated that neutral or anionic mediators bind to oxidized NR. Therefore PS was used, despite its somewhat undesirable midpoint potential, because it was the commercially available cationic mediator with a reduction potential closest to that of NR. To compensate for PS's weak redox buffering capacity in this range, it was used at moderately high concentrations. The E_m of PS is unaffected by the presence of NR (see Table 1), evidence that NR and PS do not interact nonproductively under these conditions (i.e., in a manner that does not result in electron transfer between the two molecules). The pH dependence of the E_m of NR determined in PIPES buffer between pH 6.5 and 7.5 is -38 ± 3 mV·pH unit^{-1}. This value is 8.5 mV greater in magnitude than the theoretical slope of −29.5 mV·pH unit^{-1} for each proton that binds to NR upon two-electron reduction (*49*). The slightly steeper slope is likely due either to experimental error or to the partial titration of either the reduced-state FMN or some proximate protein moiety at one end of this pH region.

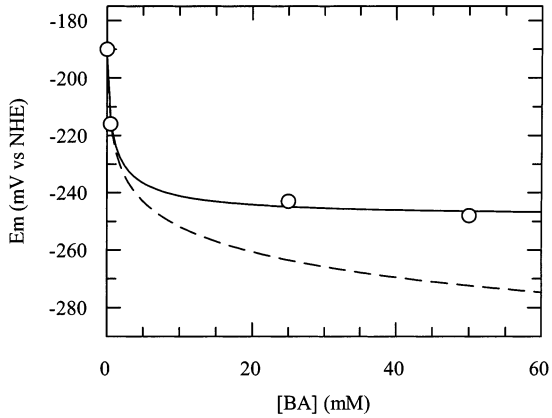

FIGURE 4: Binding of benzoic acid to reduced NR. The measured E_m values are plotted vs the concentration of BA. The solid line is a plot of eq 6 with K_{ox}, the dissociation constant for BA to oxidized NR equal to 88 μM, and K_{red}, the dissociation constant for BA to reduced NR equal to 7.6 mM. The dashed line is a theoretical curve generated using eq 6 with $K_{ox} = 88$ μM and $K_{red} = \infty$, denoting no binding of BA to reduced NR.

Unfortunately, NR is unstable beyond this pH range on the time scales necessary for by these experiments. However, this one proton−two electron slope is further evidence, along with previously reported optical spectra of the enzyme, that fully reduced NR is anionic at neutral pH.

Figure 4 shows the variation of the measured E_m of NR with the concentration of BA, a competitive inhibitor and substrate analogue (*19*, *50*). No flavin semiquinone species could be detected during the course of these experiments. As BA binding does not detectably alter the separation between the one-electron redox potentials in NR, the dependence of E_m on [BA] can be described using eq 6 (*51*) and the known dissociation constant of 88 μM for the oxidized NR−BA complex (*52*):

$$E_m = E_0 + 29.5 \text{ mV} \log\left(\frac{1 + [\text{BA}]/K_{red}}{1 + [\text{BA}]/0.088}\right) \qquad (6)$$

where E_0 is the E_m in the absence of BA (−190 mV), [BA] is millimolar the concentration of BA, and K_{red} is the dissociation constant for the reduced NR−BA complex. The

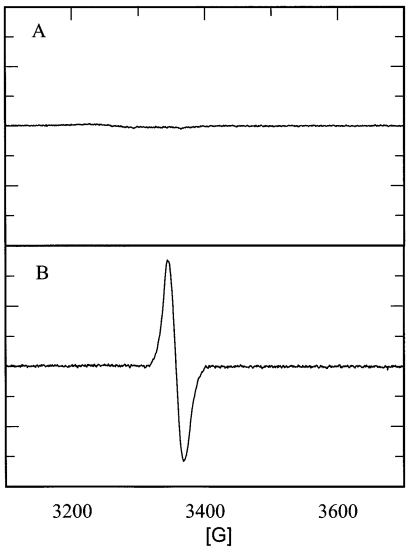

FIGURE 5: EPR quantitation of the NR semiquinone. (A) 341 μM NR in 50 mM KH_2PO_4, 50 mM KCl, 0.02% NaN_3, 1 μM PS, 1 μM MV, pH 7.0, poised at its $E_{m,7}$, −190 mV vs NHE. (B) 10.1 μM FD in 50 mM KH_2PO_4, 50 mM KCl, 0.02% NaN_3, 2 μM anthraquinone 2,6-disulfonate, 1 μM MV, pH 7.0, poised at its $E_{m,7}$, −291 mV vs NHE. As the separation between the two one-electron reduction potentials of FD is so large (−278 mV in this buffer), the concentration of flavin semiquinone radical can be assumed to be equal to that of the starting FD. As the signal-to-noise ratio in (B) is 300:1, the detection limit (at a S/N of 3:1) for FMN semiquionone is 100 nM, and the upper limit for the concentration of this oxidation state in NR poised at its E_m can be estimated as less than one part in 3400.

BA-dependent change in E_m can best be described using a value of $K_{red} = 7.6 \pm 1.6$ mM, and the limiting midpoint potential at saturating BA is −247 mV.

Electron Paramagnetic Resonance. The flavodoxin from *Desulfovibrio vulgaris* was used as a spin quantitation standard for any flavin semiquinone radical in NR because the former can be quantitatively prepared as the semiquinone (*53*). Figure 5 portrays the EPR spectra of a 10.1 μM solution of the FD semiquinone and a 341 μM solution of NR poised at its two-electron midpoint potential. As the ratio of signal to noise in the FD sample is 300:1, the detection limits of our experimental apparatus are approximately 100 nM (at a S/N of 3:1). The absence of any detectable organic radical signal in NR at its midpoint potential demonstrates that the flavin semiquinone state of the enzyme is present at a concentration of less than one part in 3400.

ApoNR−FMN$_{ox}$ Binding Assays. The redox midpoint potential reflects the difference between the binding energies of oxidized FMN and reduced FMN. To determine these energies, we have measured the dissociation constant for the binding of one oxidized FMN per monomer to the dimeric protein. Binding of oxidized FMN to apoNR quenched the intrinsic steady-state fluorescence emission of the cofactor by a factor of 3.01. Binding assays performed at starting FMN_{ox} concentrations of 99.1, 550, and 3900 nM gave a mean dissociation constant (K_D) of 8 ± 3 nM for the apoNR−FMN_{ox} complex (data not shown). The binding data were best fit to a single-site binding isotherm, indicating that the first and second FMN molecules bind to the dimer with comparable affinities (i.e., the binding is noncooperative) and that binding of any additional FMN is associated with a much weaker binding constant.

Ultracentrifugation. Ultracentrifugation was used to determine the dimerization stability of apo- and holoNR. Global fits of data at three speeds and three cell loading concentrations were best described in terms of a single species for both the apo and holo enzyme solutions in the absence of exogenous FMN. The molecular masses of the apo and holo protein species were determined to be 48 600 ± 2 500 and 49 100 ± 2 600 Da, respectively, which compare very well to the calculated dimeric molecular masses of 47 900 and 48 934 Da and establish both apo- and holoNR as dimeric at the lowest detectable concentrations. The dissociation constants for monomerization of these proteins, therefore, have upper limits of 10 nM.

DISCUSSION

The experiments described herein address the thermodynamics of protein−flavin interactions in NR: both the differential stabilization of the different cofactor oxidation states, equivalent to redox potential tuning, and the binding thermodynamics of both a first and a second FMN to a protein dimer. As these interactions determine both the thermodynamics and stoichiometry of electron transfer, they are crucial to the enzyme's as-yet undetermined physiological role.

Redox Potential Tuning in NR Enforces Two-Electron Chemistry over One-Electron Chemistry. At neutral pH, the FMN semiquinone disproportionates to form oxidized and reduced FMN in rapid equilibrium with the semiquinone, present at equilibrium as up to 8% of the total FMN (*1*, *2*). The amount of semiquinone in equilibrium with the oxidized and reduced species can be related to the difference, in millivolts, between the individual one-electron reduction potentials, E_1 and E_2, which correspond to the potentials of the oxidized/semiquinone and semiquinone/hydroquinone couples, respectively (*49*):

$$E_2 - E_1 = -59 \text{ mV} \log K \tag{7}$$

where

$$K = \frac{[\text{semiquinone}]^2}{[\text{oxidized}][\text{hydroquinone}]} \tag{8}$$

When bound to a protein, the flavin radical is generally stabilized (*51*, *54*). Relatively few flavoenzymes have been found to suppress the semiquinone to a level equal to or less than that of aqueous FMN, and of those, only two, to our knowledge, have been examined quantitatively using EPR spectroscopy (*55*, *56*). The extreme destabilization of the flavin radical in NR corresponds to a large positive gap between the first and second one-electron reduction potentials of the bound cofactor; i.e., the second reduction is significantly more favorable than the first. On the basis of the upper limit of the flavin semiquinone concentration at the midpoint potential, less than one part in 3400, the lower limit for the separation between the two potentials of NR is +381 mV. This value can be used in conjunction with the experimentally determined two-electron midpoint potential (−190 mV) to calculate an upper limit for E_1 of −380 mV and a lower limit for E_2 of +1 mV vs NHE. This limiting value of E_1 is supported by the fact that MV, which has a one-electron midpoint potential of −430 mV at pH 7.0, is an effective mediator of the dithionite reduction of NR (*49*).

NR's reduction potentials provide a basis for its ability to preferentially perform two-electron chemistry, the defining trait of a type I nitroreductase (*4*). The oxygen sensitivity of type II nitroreductases lies in the relatively facile one-electron reduction of oxygen to superoxide (*57*) by both the one-electron-reduced nitroaromatic product and the putative flavin semiquinone-containing enzyme intermediate of the reaction. The suppression of the FMN semiquinone oxidation state in NR makes the execution of one-electron chemistry by the enzyme thermodynamically unfavorable. Indeed, given the extremely broad substrate specificity of NR (*15*, *19*), a higher *K* value for its FMN cofactor would result in considerable oxidative stress for the bacterium due to the one-electron reduction of various cellular components, such as quinones. NR's suppression of its semiquinone becomes even more important in view of the recent implication of this protein family in the *soxRS* regulon, a cellular response to oxidative stress in *E. coli* (*16*). NR's inclusion in the *soxRS* regulon suggests that it provides an active defense against oxidative stress, presumably by virtue of its ability to fully reduce redox-active intracellular organic compounds (i.e., quinones, flavin derivatives, etc.) that might otherwise acquire single electrons and then generate superoxide. This protective role would be not unlike that of the mammalian DT diaphorases, a family of unrelated homodimeric flavoproteins which act to suppress both oxidative stress and basal mutation rates (*58*).

Although this relationship between reduction potentials and oxygen sensitivity is likely to apply to all enteric nitroreductases, it need not apply to all flavoproteins. For example, this relationship is not valid for the flavoprotein monooxygenases, which react with oxygen to form a covalent C(4a) hydroperoxide intermediate as opposed to merely transferring electrons and/or protons between the active-site flavin and oxygen, as in NR (*59*−*61*). In other, unrelated flavoproteins, oxygen reactivity has been shown to be modulated by substrate and/or product binding, which can change both the local environment of the active-site flavin and the stability of any superoxide product (*61*, *62*). Two arguments can be made against this form of activity modulation playing a role in the low oxygen reactivity of nitroreductases. First, these enzymes catalyze nitroreduction using a ping-pong mechanism (*19*), meaning that the reduced enzyme form that reacts with oxygen will not be complexed with either substrates or products. Second, no flavin semiquinone species were detected during the course of potentiometric titrations in the presence of the nonreactive substrate analogue benzoate, indicating that even when a substrate−NR complex exists, there is not a large alteration in the stability of this oxidation state.

The −190 mV two-electron midpoint potential of NR also explains why hydroxylaminobenzene and not aniline is the endproduct of NR's catalytic activity on nitrobenzene (*19*). Electrochemical experiments in acidic (pH $\leq$ 5) aqueous solution show nitrobenzene to be reduced at two potentials: one four-electron reduction of nitrobenzene (NB) to hydroxylaminobenzene (HAB) and a subsequent two-electron reduction of this product to aniline at potentials more than 400 mV lower (*63*). Thus, NR's inability to further reduce hydroxylaminobenzene most likely derives from its inability to generate a sufficient driving force to effect this reduction (i.e., its E_m is not low enough.)

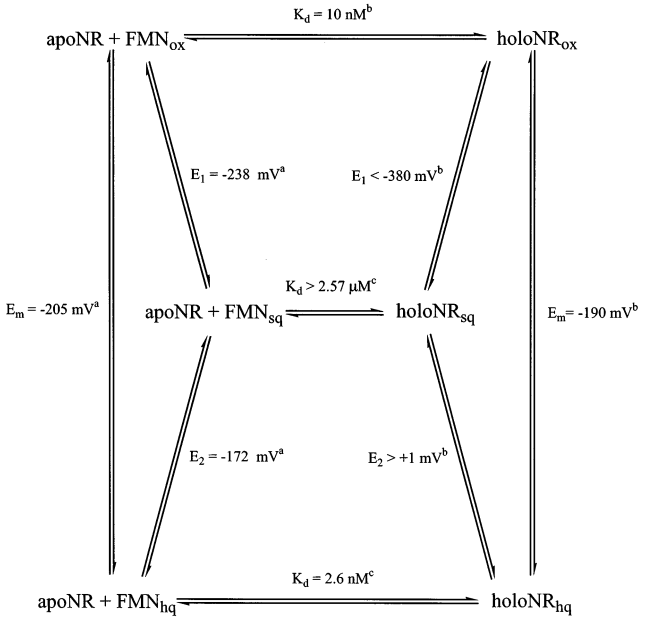

FIGURE 6: Depiction of the linked equilibria relating the midpoint potentials and dissociation constants of different apoNR−FMN complexes. FMN_{OX}, oxidized FMN; FMN_{SQ}, FMN semiquinone radical; FMN_{HQ}, two-electron-reduced FMN hydroquinone. [a]-Experimental values determined by Draper and Ingraham (*1*). [b]-Experimental values presented in this work. [c]Values calculated using thermodynamic cycles as described in the text.

Benzoate is a competitive inhibitor vs NADH which binds tightly to oxidized NR (*52*). A single molecule of benzoate binds over the *re* face of the pyrimidine and central rings of the isoalloxazine in a manner believed to be analogous to binding of the nicotinamide moiety of NADH (*31*, *50*). The binding site may also represent the site of nitroaromatic binding, as the benzoate molecule contacts residues shown in the *E. coli* homologue NfsA to modulate oxidizing substrate specificity (*64*). The relatively weak binding of benzoate to fully reduced NR and the lower E_m value of the complex are likely due to electrostatic repulsion between the anionic flavin hydroquinone and benzoate. Indeed, reduction of crystals of the acetate complex of NR caused acetate to be released, in conjunction with only a relatively minor structural change which does not appear as if it should sterically hinder binding (*31*).

The Relation of Flavin Binding to Redox Potential Tuning in NR. The oxidation potentials presented here and the free energy of oxidized FMN binding to apoNR can be used in simple thermodynamic cycles to calculate NR's affinities for FMN in its different oxidation states (see Figure 6) (*65*, *66*). On the basis of the limiting values calculated for the two individual one-electron reduction potentials, the upper limit for the stability of the flavin semiquinone−apoNR complex can be estimated to be at least 257-fold weaker than the stability of the oxidized flavin-apoNR complex.[2] Schopfer et al. have proposed that the stability of the flavin radical may be controlled by proteins in part via alterations in the pK_a at N(5) of the semiquinone (*67*). This postulate has been supported by recent discoveries regarding flavin semiquinone

[2] This value has been calculated using the free flavin redox potentials of Draper and Ingraham (*1*). The exact values of these potentials are still under debate, and some authors, in particular Mayhew, have recently proposed a wider separation of potentials for free FMN (*2*).

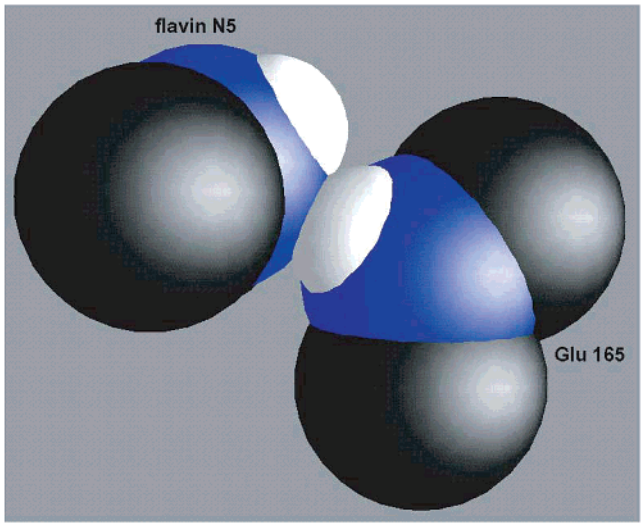

FIGURE 7: Space-filling representation of the interaction between N5 of the reduced flavin and the main-chain amide nitrogen of Glu 165. The figure was made with GRASP (*80*).

stabilization in *Clostridium beijerinckii* flavodoxin (*68*). In this flavoprotein, the large *K* value of over 150 000 has been attributed in great measure to a hydrogen bond between the N(5) proton and the backbone carbonyl oxygen of glycine 57, which favors protonation of N(5) and thus stabilizes the neutral semiquinone oxidation state of the cofactor (*66*, *69*). In NR, protonation at N(5) appears to be disfavored by hydrogen bond donation *from* the backbone amide proton of glutamate 165, although the limited pH stability of the enzyme precludes any direct experimental verification of the N(5) pK_a. The backbone amide proton is in van der Waals contact with the *si* side of N(5) in the reduced state (see Figure 7), indicating that the flavin must increase its degree of bending in order to accommodate the additional proton of the anionic hydroquinone. Thus, the NR structure could sterically decrease the pK_a at N(5) and destabilize both the neutral flavin semiquinone and the hydroquinone.

We also note that the oxidized flavin in NR has an unusually large (16°) "butterfly" bend angle about the N(5)−N(10) axis which increases to 25° upon full reduction. While a role for ring strain in the modulation of two-electron potentials has been a source of contention (*70*−*72*), a role in the (de)stabilization of the flavin semiquinone has not been addressed experimentally. A number of theoretical studies indicate, however, that flavin semiquinones are planar (*73*−*75*, *81*, *82*). In light of this, it is interesting to compare the active site of NR with that of old yellow enzyme (OYE.) The flavin cofactor in OYE interacts with its protein binding site by a contacts very similar to those in NR, including amide hydrogen bond donation to N(5), but the flavin adopts an almost planar conformation in the oxidized state (*76*). The fact that OYE has a relatively stable semiquinone, with a +30 mV separation between E_1 and E_2 instead of the >+381 mV separation in NR (*77*), suggests that the extreme bend angle in NR could be an important contributor to semiquinone destabilization. The enforced bending in both the oxidized and hydroquinone states (and thus most likely in the unobservable semiquinone as well)[3] would destabilize the oxidized and semiquinone states. Indeed, the two possible mechanisms mentioned above might act in concert, with the hydrogen bond from the backbone amide of Glu165 forcing a hypothetical neutral semiquinone to be even more bent than the oxidized flavin conformation, to accommodate the additional proton at N(5). They might also conspire to destabilize the semiquinone in particular: flavin bending is expected to destabilize both the oxidized and semiquinone oxidation states, but hydrogen bond donation to N(5) is expected to stabilize the oxidized state while destabilizing the reduced and neutral semiquinone states, with the net effect being that only the semiquinone state is destabilized on both counts. The contributions of these structural properties to the redox thermodynamics of NR are currently being assessed by a combination of site-directed mutagenesis, computation, and NMR spectroscopy.

FMN Binding and Dimerization Equilibria. The crystal structure of NR, with the two non-covalently bound flavin cofactors sandwiched between the two protein monomers, suggests that monomer−dimer equilibria and flavin binding events may be related, greatly complicating the thermodynamics of the NR−FMN complex. Indeed, both our preliminary investigations (*78*) and recent work performed by Liu et. al on the distantly related *Vibrio harveyi* NADPH−FMN oxidoreductase (*79*) supported this hypothesis. However, both the concentration independence of the FMN dissociation constant and the analytical ultracentrifugation results demonstrate that, in the absence of exogenous FMN_{ox}, both holoNR and apoNR are dimeric at concentrations comparable to those found in vivo in the strain of *Enterobacter* from which NR was cloned (*14*).

In conclusion, we have measured the fundamental thermodynamic properties of NR, and they agree well with the observed reactivity of the enzyme. The limits this work places on E_1 and E_2 can explain NR's identity as an oxygen-insensitive nitroreductase, and the measured E_m is consistent with NR's reduction of NB to HAB but not aniline. Inspection of the crystal structures of NR has led to some novel hypotheses concerning the structural origin of these thermodynamic properties, which are currently under experimental investigation.

[3] Especially in light of the inflexibility (average crystallographic *B* factor of less than 12 Å^2) of the flavin binding site in the oxidized and reduced crystal structures (*31*).

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